Evaluation of an *in vitro hsp70* induction test for complex mixtures toxicity assessment: comparison with chemistry and ecotoxicity tests

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ABSTRACT

The aim of this study was to assess the potential of a human cell line containing the *hsp70* promoter linked to the chloramphenicol acetyl transferase reporter gene, to evaluate toxic potential of complex mixtures. Cells were exposed to eluates of industrial wastes and the cellular responses were compared to the metal contents of the samples and to standardised aquatic (microalgae growth inhibition, daphnia immobilisation, bacterial luminescence inhibition, *Ceriodaphnia dubia* reproduction inhibition) and terrestrial (earthworms lethality, plant growth inhibition) tests. The *hsp70* promoter was significantly induced by 11 out of 14 samples, with different dose-response patterns. Significant correlation of *in vitro* induction potency with aquatic ecotoxicity, especially with chronic tests, and with the metal contents of the samples were observed. Our study provides new information on the relevance of *hsp70* gene induction as a criterion of toxicity and suggests its usefulness for the detection of toxicity associated to metallic pollution in complex mixtures.

Key Words: industrial wastes; heat shock protein; reporter gene; cellular stress; ecotoxicity tests; dose-response modelling; multivariate comparison.

INTRODUCTION

Hazard evaluation of environmental complex mixtures consists in the determination of their intrinsic toxicity towards living systems and thus requires information given by both chemical analysis and toxicity testing. It has now become evident that chemical analysis is not sufficient to achieve hazard assessment of environmental samples, since it is restricted to some specific classes of toxic compounds, for which analytical methods are available and toxicity is known. In addition, chemical analysis do not inform neither on the available fraction for living organisms nor on the potential joint effects of the different contaminants. Hence, a toxicological evaluation using bioassays has to be done in order to determine the actual hazardous properties of a given sample.

The potential of *in vitro* approach using cell tissues for assessing the harmful effects of chemicals on living systems has recently attracted much attention in ecotoxicology (reviewed by Walker, 1998; Wells et al., 1998). An emerging trend concerns the *in vitro* use of biochemical and molecular biomarkers for toxicity testing and the measurements of cellular responses related to the mechanisms of toxicity (Bierkens *et al.*, 1998; Walker, 1998; Wells *et al.*, 1998). In particular, cellular assays using reporter gene coupled to stress inducible gene promoters have gained acceptance as valuable tools for toxic potential evaluation. They are generally sensitive and specific, and their relative easy use and cost effectiveness allow their use in screening programs.

At present, the application of receptor-mediated gene responses to environmental mixtures has been well studied with regard to the detection and quantification of specific contaminants such as dioxin-like compounds (Anderson *et al.*, 1995; Kerr *et al.*, 1999) or xenoestrogens (Zacharewski,

1997; Balaguer *et al.*, 1999). The validity of the biological response has been achieved by correlating reporter gene induction with chemical analysis of the samples (Anderson *et al.*, 1995; Vincent *et al.*, 1997; Kerr *et al.*, 1999). Besides, the application of cellular damages-mediated reporter genes, such as those involved in response to heat shock, oxidative stress and/or DNA alteration, has been less extensively characterised for toxicity assessment of environmental mixtures, although several studies have reported their activation by environmentally realistic pollutant levels (Fischbach *et al.*, 1993; Guven *et al.*, 1994; Van Dyk *et al.*, 1994; Jones *et al.*, 1996) or environmental samples (Vincent *et al.*, 1997; Power *et al.*, 1998; Hamers *et al.*, 2000). These cellular responses are activated by stressors that alter vital cellular functions (*i.e.* proteins or DNA alteration), and are generally associated to early (cyto)toxic events (Goering *et al.*, 1993; Neuhaus-Steinmetz and Rensing, 1997; Aït-Aïssa *et al.*, 2000). Thus, they may represent potential predictors of toxic effects at higher levels of biological organisation. However, to our knowledge, no study compared such *in vitro* stress gene induction assays with *in vivo* (eco)toxicity effects. Unlike several *in vitro* cytotoxicity tests using primary or continuous cell cultures which have been shown to correlate to *in vivo* chemicals toxicity or ecotoxicity assays (Saito *et al.*, 1993; Segner and Schüürmann, 1997; Gagné and Blaise, 1998), the predictive information provided by cellular reporter gene tests is left unexplored.

In this paper, we address the use of heat shock gene (*hsp70*) induction in response to exposure to complex mixtures in a stably transfected cellular model. The heat shock response is characterised by the induction of a set of stress proteins, namely called heat shock proteins (HSPs). Under physiological conditions, HSPs act as molecular chaperons and ensure cellular protein homeostasis and cell protection. After cellular aggression, the release of the heat shock transcription factor (HSF) from its binding to HSP70 in the cytoplasm, allows formation of an

HSF homotrimer which migrates to the nucleus and binds the heat shock elements (HSE) in the promoter region of *hsp* genes, thus leading to the transcription activation of these genes. It is know admitted that the stressor-induced generation of misfolded proteins constitutes the trigger signal that up regulates the heat shock response (Hightower, 1991), and a number of environmental pollutants were shown to induce HSPs synthesis in many organisms or cell tissues (Sanders and Martin, 1993), through different mechanisms of action involving cellular protein alterations (Voellmy, 1996). Due to their strong conservation through evolution, the wide diversity of their inducing agents and the relative sensitivity of their expression in comparison to conventional endpoints such as growth, survival or reproduction, the HSPs, and especially the major stress protein HSP70, have been proposed as sensitive markers of non-specific effects in environmental monitoring (Sanders, 1993; Ryan and Hightower, 1996; Bierkens, 1998).

In a previous study, we used HeLa cells carrying the human *hsp70* promoter linked to the chloramphenicol acetyl transferase (CAT) reporter gene (Kretz-Remy and Arrigo, 1994) to screen toxic chemicals (Aït-Aïssa *et al.*, 1999; Aït-Aïssa *et al.*, 2000). Heavy metals, pesticides, and chlorophenols induced the *hsp70* promoter in this model, whereas some organic chemicals were found negative even at cytotoxic doses. The authors concluded that although HSP70 should not be considered as a universal marker of toxicity, it might be useful for the detection of toxic effects generated by many different types of chemical contaminants in complex samples. The aim of the present study was (i) to evaluate the applicability of the established *hsp70*-reporter gene model to reveal biological activity in complex mixtures, and (ii) to assess its toxicological significance by investigating how this cellular test compares to a battery of ecotoxicity tests and to chemical analyses. For this purpose, HeLa-CAT cells were exposed to fourteen eluates of industrial wastes and the cellular responses were compared to the metallic contents of the samples and to

ecotoxicological effects, as measured by standardised aquatic and terrestrial tests performed in parallel on the wastes (Pandard, 2000).

MATERIALS AND METHODS

Samples description, preparation and chemical analysis

The table 1 summarises the characteristics and the origins of the industrial wastes used in this study. Sampling, waste description and sample preparation have been detailed elsewhere (Pandard, 2000). Briefly, eluates of wastes were prepared by mixing the raw waste with distilled water with a liquid to solid (dry matter) ratio of ten, for 24 hrs under circular agitation at room temperature, according to the draft european standard (prEN12457-2). After settling, the supernatant was collected and filtered through a 100 µm size pore filter. The pH was then adjusted between 5.5 to 8.5 if pH value was out of this range. The samples were filtered again through a 0.45 µm filter and then used for toxicity testing and chemical analysis. All aquatic ecotoxicity tests were performed on the 0.45 µm filtered fraction except the *Daphnia magna* acute test (see below) which was performed on the 100 µm filtered fraction. For *in vitro* testing, a further filtration of the samples through 0.2 µm filters was performed in order to avoid any bacterial contamination of the cultures. For metal analysis, the 0.45 µm filtrates were analysed by inductively coupled plasma atomic emission spectroscopy (ICP/AES) according to standard procedures (NF EN ISO 11885, NF EN 1483 and NF T 90-043).

In vitro cellular stress test

HeLa-*hsp70*-CAT cells were routinely cultured in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated foetal bovine serum (Gibco, France) and 1% antibiotics (Penicillin-Streptomycin) at 37°C in a humidified atmosphere containing 5% CO_2. Cellular test conditions were the same as those previously described for chemical compounds testing (Aït-Aïssa *et al.*, 2000). Briefly, subconfluent cells (about 80% confluency) grown in 24-wells culture plates were washed twice with pre-warmed PBS and exposed in triplicates to various concentrations of the samples, ranging from 0.1 to 90% v/v. Just before exposure, nine volumes of sample were mixed with one volume of ten fold concentrated phosphate buffer saline (PBS), giving the upper range concentration (90 %), and thereafter serially diluted in serum-free culture medium. The former step allowed a salt correction of the sample as well as a pH stabilisation in physiological range values, thus preventing any osmotic or pH stress to the cells at the highest concentrations. Preliminary experiments showed that a 24 h exposure of cells in PBS did not neither modify the cell viability nor activate significantly the stress promoter. After a 16 hr exposure to wastes eluates, the cultures were washed once with pre-warmed complete medium and allowed to recover for 24 hours in complete medium before harvesting. Analysis of intracellular CAT content was performed with a CAT ELISA kit (Boeringher Mannheim) according to the manufacturer's instructions. Results are expressed as the ratio between the amount of CAT detected and the total cellular protein content as determined by a modification of the Lowry's method (BioRad *DC* Protein Assay kit). Cytotoxicity was evaluated by the total protein assay and was expressed as percentage of control cultures. Samples were stored at 4°C in the dark and tested for cellular stress 3 days maximum after preparation. This storage time did not

influenced their toxic potential since we observed that a storage for two weeks did not diminish significantly their *in vitro* toxicity (not illustrated).

Ecotoxicological tests

The ecotoxicity tests (Table 2) were performed according to standard procedures. Aquatic tests were performed either on the 100µm (*Daphnia magna* test) or on the 0.45µm (*C. dubia, P. subcapitata* and *V. fischeri* tests) filtered fractions. For all three terrestrials tests, dilutions of raw wastes were carried out in artificial soil (20 % Kaolinite clay, 10% sphagnum peat finely ground, 70 % fine quartz sand with more than 50 % of particle size between 0.02 mm and 0.5 mm).

Analysis of hsp70 induction data

Hsp70 induction curves parameters, such as effective concentrations, maximal induction level and the rate of the induction, were calculated after modelling the observed *in vitro* patterns. Because *hsp70* induction profiles were often balanced by a cytotoxic effect, thus leading to biphasic induction curves, the measured effects were modelled as follows. Firstly, cytotoxicity data, expressed in % of control cultures, were fitted to a logistic model according to equation (1) :

$$(1)\quad y = \frac{A - D}{1 + \left(\frac{concentration}{EC_{50}} \right)^{B}} + D$$

where y = (cyto)toxicity, expressed in % of control, A = no effect value (set to 1), B = parameter representative of the slope of the viability fall, EC_{50} = concentration leading to 50% (cyto)toxicity compared to control and D = maximal effect value (set to 0 for 100% toxicity). The resulting

calculated parameters were then reported on a second model combining two logistic curves. The equation for the observed induction becomes:

$$(2)\ induction = Equ.(1) \times \frac{A'-D'}{1+\left(\frac{concentration}{C'}\right)^{B'}} + D'$$

where A' = level of CAT in control, B' = parameter representative of the slope of the induction curve in absence of cytotoxicity interference, C' = concentration leading to median induction (*i.e.* in absence of cytotoxicity interference) and D' = maximal induction level in absence of cytotoxicity.

The coordinate values of the maximum induction (Xmax, Ymax) were calculated from the first derivative of equation (2). The induction slope characteristics (maximal slope value at the inflection point of the increase and its corresponding concentration (EC_{slope})) were calculated from the second derivatives of equation (2). All the calculations were performed on Microsoft® Excel spreadsheets by least squares minimisation.

Analysis of in vivo toxicity data

We aimed to use a single endpoint for each ecotoxicity test in order to reduce the number of variables to be further introduced in the multivariate analyses. The EC_{20} (concentration leading to 20% of effect as compared to control) was chosen because it relies to a low effect and thus appeared more adapted than a EC_{50} to compare sublethal cellular effects to *in vivo* acute and chronic effects. Dose-response curves from ecotoxicity tests were modelled using equation (1). The EC_{20}s were calculated from the following equation :

$$(3)\ EC_{20} = EC_{50} \times \left[\frac{(A-D)}{(0.8-D)} - 1\right]^{\left(\frac{1}{B}\right)}$$

where A, B, EC_{50}, D are calculated parameters resulting from equation 1.

Statistics

In vitro effects – Significant effects of wastes on either *hsp70* induction or total protein content were tested by ANOVA. Homogeneity of variances was analysed with the Levene's test. When variances were found homogenous, significant effects of the tested concentrations on either *hsp70*-CAT induction or cytotoxicity were determined by a one-way ANOVA, followed by an unilateral Dunnett's test. When variances when not found homogenous, a non parametric Kruskall-Wallis test was performed. A value of $p<0.05$ was considered statistically significant.

Distribution of the chemical and toxicity data - The Lilliefors test, based on a modification of the Kolmogorov-Smirnov test, revealed that none of the datasets were normally distributed. Therefore, the data were log-transformed to conform to the normality assumption (Lilliefors test) and to the homogeneity of variance (Levene's test).

Principal component analysis (PCA) - The degree of similarities between all toxicity tests used for the characterisation of the industrial wastes was explored by running a principal component analysis (PCA) on log-transformed data. The PCA was run from a data matrix which included the eight toxicity variables (four aquatic, three terrestrial and the *hsp70* test) as the columns and the fourteen wastes as the lines. Chemical data were not included into the PCA due to missing values.

Correlation analysis - Correlation between stress promoter induction and either chemical analysis or *in vivo* toxicity were determined with the Pearson correlation on log-transformed data. Significance of the correlation was determined with a two-tailed Student's *t* test for significance. All statistical calculations were carried out with SPSS® 10.0 for Windows.

RESULTS

In vitro stress responses and calculation of induction potencies (PI)

The table 3 summarises the *hsp70* activation and cytoxicity results. Eleven samples out of 14 induced significantly ($p<0.05$) the *hsp70* promoter in a concentration-response relationship. Different degrees of cellular stress response were noticed when considering the level of the CAT production and the effective concentrations. Some wastes, *i.e*. wastes 7, 8 or 11, induced a strong gene response at relatively low concentrations. Others, *i.e.* wastes 1, 2, 5 and 14 gave intermediate levels of response, while the last samples provoked low (wastes 3, 10, 12, 13) or no (wastes 4, 6, 9) activation of the promoter. On a first approach, these results show a good responsiveness of the cellular model for the detection of biological effects associated to complex mixtures, allowing a first ranking of the samples in three groups. In order to standardise the results, the measured effects were fitted to a double logistic model, as described in the Materials and Methods section. Examples of fits which illustrate different patterns of *hsp70* induction by complex mixtures are given in the figure 1. As seen in this figure, this mathematical model allows a good fitting of the measured effects by non linear regression.

In order to rank the wastes with respect to their potency to induce the *hsp70* promoter, we aimed to reduce the individual dose-response curves to simple descriptors which should reflect the induction potency (PI) of a given sample. Because the observed induction profiles varied in function of two parameters, *i.e.* the level of CAT production and the concentration of sample responsible for this induction, the PI is defined as the intensity of the reporter gene response per sample concentration unit. Hence, PI value could be represented either by the slope of the induction, which reflect the rate of gene induction with respect to the concentration, or, as

previously described (Aït-Aïssa *et al.*, 2000), by the ratio between the maximal induction value and the concentration leading this maximal induction (Ymax/Xmax). With our present set of data, a high correlation ($r = 0.99$, $p<0.001$) was obtained between the slope of the induction and the Ymax/Xmax values, suggesting that these two variables are equivalent. In further analysis, we used two parameters to describe the dose-response curves : the Ymax/Xmax variable as an index for *hsp70* induction potency, and the EC_{slope} endpoint (concentration responsible for median induction) in order to allow direct comparisons of effective concentrations measured in the different tests.

Comparison with metal contents

Values for selected metals are summarised in Table 4. Although only partial data were available, it appears clearly that metal contents presented a great diversity amongst the analysed samples. High levels, *i.e.* above a milligram per litre, were found for zinc, cadmium, copper and lead in samples 5, 7, 8 and 11. Oppositely, very low to not detectable concentrations were found in wastes 3, 4, and 6. The total elemental contents of the other wastes (1, 2, 9, 14) were found in the 0.1-1 mg/l range. While it is interesting to note that wastes 5, 7, 8 and 11 came from metal industry, no clear evidence enabled us to rely the chemical characteristics of a given sample to its original industrial sector.

The *hsp70* induction potencies of the samples were generally consistent with the chemical data. The relationships between chemical data and *in vitro* data were analysed by linear regression, performed on log-transformed PI versus log(1+metal), with the metal contents expressed in $\mu g.l^{-1}$. When considering individual elements, PI was significantly correlated to cadmium ($r = 0.904$; $p<0.001$), zinc ($r = 0.869$; $p = 0.001$) and lead ($r = 0.915$; $p<0.001$). This relationship was also

true when considering the sum of cadmium, zinc and lead ($r = 0.918$; $p < 0.001$). Very similar results were obtained when comparing the EC_{slope} factor to metal contents, significant correlation being observed with cadmium ($r = -0.900$; $p<0.001$), zinc ($r = -0.812$; $p = 0.002$) and lead ($r = -0.879$; $p<0.001$). No significant correlation was noticed with the other individual metals, nor with unspecific parameters such as total organic carbon or conductivity of the samples (not shown). The relationships between *hsp70* potencies and sum of cadmium, zinc and lead is illustrated in figure 2. These findings confirm the hypothesis that *hsp70* induction is explained by the presence of metals in the samples.

Comparison with in vivo ecotoxicological effects

A battery of terrestrial and aquatic ecotoxicity bioassays has been performed on the raw wastes and on their corresponding eluates respectively. Because *hsp70* induction relies to an effect at the subcellular level, we have chosen to compare it with a low effect endpoint, *i.e.* EC_{20}, measured in all *in vivo* tests. The measured *in vivo* toxicity and the *in vitro hsp70* induction are summarised in the Table 5.

The diversity of the *in vivo* wastes toxicity appears clearly in the Table 5. In order to explore the degree of similarities between the toxic responses given by the different tests, we ran a PCA normalised on the log-transformed data. In order to include all wastes into the calculation, we used 100% as EC_{20} or EC_{slope} values for the non toxic measurements. The locations of the samples and the tests on the first factorial plan resulting from the PCA are presented in Figure 3A and 3B, respectively. The PCA showed that 86 % of the overall variance is explained by the first two principal components. The first factor (F1) corresponds to 75 % of the overall variance and can be interpreted as a toxicity axis. For instance, wastes 8, 11 and 7 which are highly toxic for

most of the organisms, are located at the left side of the figure, at the opposite of the non toxic wastes 4, 6 and 12 (Figure 3A). All the tests, including the *hsp70* one, are able to distinguish between very toxic and non toxic samples since they present similar levels of correlation with the first component (Figure 3B). F1 is positively correlated with all ecotoxicity tests and with the EC_{slope} and negatively correlated with PI. This last negative correlation can be explained by the difference of expression unit between PI and ecotoxicity tests, *i.e.* a toxic waste will have a low EC_{20} and a high *hsp70* induction potency. The second factor (F2) corresponds to 11 % of the global variance and distinguishes between terrestrial and aquatic tests (Figure 3B). This is likely due to differences in the samples tested in either aquatic or terrestrial tests, *i.e.* eluates or raw wastes respectively, thus leading to differences in bioavailable toxic compounds. For instance, the wastes 8, 10 and 5 were more toxic for the aquatic than the terrestrial organisms (Table 5). For these samples, the leaching step may have had a strong influence on the release of water soluble toxic compounds on the bioavailable fraction. Oppositely, waste 13 was more toxic in terrestrial than in aquatic tests. This waste came from petroleum industry and contained significant levels of polyaromatic hydrocarbon (PAHs) compounds, as confirmed by HPLC analysis (not shown). The presence of such hydrophobic toxicants in the raw waste explains why the water soluble fraction was less toxic than the solid phase, where PAHs were bioavailable for terrestrial organisms only.

Figure 3B shows that the *hsp70* test was more closely related to aquatic than to terrestrial tests as EC_{slope} is located in the aquatic tests group and PI is inversely correlated to this group on the second axis of the first factorial plan. This was confirmed by the univariate correlation analyses presented in Table 6. The Pearson correlation show that PI was highly correlated ($p<0.01$) with the two chronic tests *P. subcapitata* and *C. dubia* and with the acute test *D. magna*. Lower correlation, but still significant ($p<0.05$), were obtained with the bacterial *V. fischeri* test and with

the three terrestrial tests. Correlation with the EC_{slope} endpoint were very similar to those observed with PI, although the level of significance with acute tests were slightly higher with the EC_{slope}.

Correlation analysis were performed from all toxicity measurements, including the arbitrary values of the non toxic results (i.e. EC_{20} = 100 %). However, it is likely that the high observed correlation may be due to an artefact of our dataset introduced by extreme values, *i.e.* non toxic or highly toxic values. To verify this hypothesis, a second set of correlation analysis was run without introducing the *in vitro* and *in vivo* non toxic results into the analysis. The results presented in Table 7 show that correlation between PI or EC_{slope} and aquatic tests (CER, ALG and DAP) were still highly significant, despite a lower number of samples were taken into account. The association between PI and these aquatic tests is illustrated in Figure 4. Oppositely, the correlation coefficients between PI and the bacterial and terrestrial tests were highly diminished, thus confirming that, with these last tests, an artefactual direction toward a region of non toxic values was established when all samples were introduced into the analysis. This was also true with the EC_{slope} endpoint (Table 7), excepted with the acute test with earthworm for which a significant linear relationship was still noticed after excluding non toxic values.

DISCUSSION

Cellular stress responsive reporter gene systems have been proposed as useful tools for toxicity potential evaluation in biomonitoring. However, few studies have reported their application to complex mixtures, or have compared *in vitro* stress gene response with (eco)toxicity endpoints,

measured at the cellular or multicellular levels. In the present study, we report the applicability of this cellular model for the determination of toxic potential of complex mixtures such as eluates of industrial wastes coming from different industrial sectors or processes. The relevance of the cellular stress response as a tool for toxicological diagnostic was assessed by comparing the *in vitro* results to (i) chemical analysis of the samples and (ii) *in vivo* ecotoxicological measurements from four aquatic and three terrestrial tests performed in parallel on the wastes (Pandard, 2000).

In vitro results

For 11 out of 14 analysed samples, a significant induction of the promoter was observed in a dose-response relation, with various levels of gene response intensity and effective concentration. The intensity of the response is an important parameter to be considered for the characterisation of *hsp70* induction pattern. It strongly varies in function of the type of the inducing agent (Fischbach *et al.*, 1993; Sacco *et al.*, 1997; BourniasVardiabasis *et al.*, 1998), and several studies showed that intracellular HSP accumulation can reach very high levels after severe treatment (Dreano *et al.*, 1986; Lindquist and Craig, 1988; Sanders, 1993).

By modelling the induction dose-response curves, we were able to calculate an index for *hsp70* induction (PI) and thus to classify the samples with regard to their potency to activate the *hsp70* response. In preliminary works, we have tested other non linear regression methods such as a combination of linear and logistic regression, Gaussian (Kennedy *et al.*, 1996) or exponential (Vincent *et al.*, 1997) models, but graphical analysis showed that our model provided the best fit of our data. The model we retained allowed us to calculate both an induction potency (PI) index for a given waste and the concentration of eluate responsible for median *hsp70* induction (EC_{slope}). The PI index combines both the magnitude of the gene response and the effective

concentration of stressor. A similar index, which reflected the rate of change of marker protein with the log value of the concentration, was developed by Vincent *et al.* (1997) to describe induction potency of a given stressor. The authors used this parameter to compare stress gene response to chemical data in recombinant HepG2 cells exposed to air particles suspensions. This allowed them to identify substances well correlated to stress gene response. For instance, they identified amongst 21 analysed metals boron, titanium, copper and zinc as possibly associated with the cellular responses, while soluble copper emerged as the best explanatory variable for *hsp70* and metallothionein promoters induction.

Hsp70 versus analytical data

Our results are in line with those previously reported by Vincent *et al.* (1997), since *hsp70* induction in HeLa-CAT cells were consistent with the presence of metals in the samples (Figure 2). Heavy metals are well known as transcriptional activators of HSP70 in a plethora of living systems (Fischbach *et al.*, 1993; Sanders, 1993; Guven *et al.*, 1994; Ryan and Hightower, 1994). Although partial chemical analysis were performed, the *hsp70* response is explained, at least partly, by the contents in cadmium, zinc and lead (Figure 2). For instance, wastes 7, 8 and 11 were the most efficient *hsp70* activators ; they also contained the highest amounts of metals, mainly cadmium, zinc, lead and copper. These metals, with zinc to a lesser extent, have been ranked amongst the most efficient chemicals able to induce the *hsp70* promoter in different reporter gene systems (Fischbach *et al.*, 1993; Guven *et al.*, 1994; Steiner *et al.*, 1998). On the opposite, metal contents in the wastes 4, 6 and 9, were very low to not detectable. In the same way, these samples were not effective to induce any significant stress response, even if a clear cytotoxic effect of the sample 9 was observed. The waste 5, which induced a moderate response

of the *hsp70* gene, did not fit well into the correlation (Figure 2). This sample had a relatively high zinc content but was less potent to induce the *hsp70* promoter than wastes 1 and 14, which contained ten times less metals. This observation suggests that, although a significant linear correlation was noticed between PI and chemical analyses, induction of stress response by metals mixture may be the reflect of complex mechanistic events involving chemicals interactions and thus cannot be completely explained by simple linear relationships with individual or addition of metals species.

Metals ions are known to exert interactive effects when they are present in mixture (Newman and McCloskey, 1996), however it is not clear whether these effects are additive, antagonistic or synergistic. *In vivo*, this might depend on various conditions such as the nature of metal considered, the tested concentrations or the studied organisms (Steiner *et al.*, 1998; Sharma *et al.*, 1999; Preston *et al.*, 2000). In addition, only few data exist with *in vitro* systems. Recently, Tully *et al.* (2000) reported the ability of toxic metals, alone or in combination, to induce 13 stress related promoters, including *hsp70*, in recombinant HepG2 cells. No synergistic activity was detected after exposure to an environmentally relevant mixture of cadmium, lead and chromium (III). By using the parental cell line HepG2, Steiner *et al.* (1998) showed different interactions between metals with regard to their abilities to stimulate *hsp70* mRNA production. They observed antagonistic effects on *hsp70* induction by paired combinations of metals such as cadmium/zinc or cadmium/manganese, and a potentiating effect, coupled to cytotoxicity, after a concomitant exposure to mercury and silver. Therefore, considering overall or individual metals of a complex mixture should be only partly explanatory for *hsp70* induction since some are more effective than others to induce the stress response and interactions between elements may occur. Nevertheless, integration of chemicals interactions in the biological response constitutes one

major argument for the preferential use of bioassays rather than chemical analysis for the determination of toxic potential. Such argument is in line with the view that "stress protein accumulation may reflect the intregrated stress load on the organism regardless of the type or number of stressors involved" (Sanders, 1993).

Comparison with in vivo ecotoxicity

One major aim of this study was to determine how comparable the *hsp70* test and a set of ecotoxicity tests are. PCA showed that all the tests, including the *in vitro* one, were able to distinguish between highly toxic and non toxic samples (Figure 3). Furthermore, significant correlations were observed between PI and *in vivo* ecotoxicity of wastes (Table 6). When excluding non toxic results, PI was correlated to the *C. dubia* and *P. subcapitata* chronic tests ($p<0.01$), and to a lesser extent ($p<0.05$), to the *D. magna* acute test (Table 7, Figure 4). Similar relationships were observed between aquatic tests and EC_{slope} factor, although some slight differences may be underlined. For instance, the PI descriptor, which relies to the intensity of the stress gene response, appeared more related to chronic tests than was EC_{slope}. This suggests that the magnitude of such molecular effect may provide additional toxicological information than the knowledge of toxic concentration only. However, more data will be necessary to validate this hypothesis.

In general, our results show that, although a limited number of samples were analysed, a clear linear relationship between *in vitro* responses and *in vivo* aquatic toxicity was revealed for this set of samples. To our knowledge, this constitutes the first report on such *in vitro*/*in vivo* correlation using a cellular reporter gene test. Besides, similar relationships between *in vitro* cytotoxicity results and acute *in vivo* ecotoxicity tests results were often reported for a set of selected

chemicals (Saito *et al.*, 1993; Segner and Schüürmann, 1997) or environmental wastewaters (Gagné and Blaise, 1998). Such good correlations support the hypothesis of basal cytotoxicity, which stipulates that toxicity findings can be extrapolated between systems if chemical toxicity is caused by injury to basal structures and functions that are common to cells of all organisms and species (Segner and Schüürmann, 1997). The heat shock response is induced in a protective way through non specific mechanisms of toxicity which involve the generation of abnormal proteins and alteration of cellular functions. As a consequence, maximum HSP induction is often related to early cytotoxic but reversible events (Goering *et al.*, 1993; Neuhaus-Steinmetz and Rensing, 1997). In our study, *hsp70* induction was generally more sensitive than cytotoxicity (up to 25 fold for waste 1) and gave additional information on the molecular mechanisms of toxicity as well as on the extent of the cellular damages that occurred. Interestingly, our results suggest that *hsp70* induction potency is more predictive of sublethal (*i.e.* chronic tests) than of acute *in vivo* effects.

The multivariate analysis revealed a low species specificity for this set of samples. This observation is unusual in ecotoxicology testing since the chosen test organisms are often phylogenetically far from each other. For these samples, the toxic mechanisms involved in the measured effects were not specific to the species. Nevertheless, this may be due to some inherent limitations of our dataset, *i.e.* a matrix of data of 14 samples to 9 variables is not propitious to obtain a good discrimination of the variability between tests. Therefore, it must be kept in mind that our positive results were obtained from a low number of samples, mainly contaminated by toxic metals. More data with other types of environmental mixtures (e.g. effluents, contaminated soils extracts, sediments, etc) will be necessary to improve the limit of the application of this *in vitro* test and to further validate its application in environmental toxicology. Finally, the use a battery of different molecular sublethal endpoints should allow the detection of a broader range of

xenobiotics which act through various mechanisms of toxicity (Blaise *et al.*, 1998) and would be more suitable for an exhaustive environmental screening.

CONCLUSION

In summary, this study reports for the first time the potential of a reporter gene test as a possible predictor of *in vivo* ecotoxicity of complex mixtures contaminated by metals. The significant correlation obtained between *in vitro hsp70* promoter induction and chemical or ecotoxicological data suggest that the reporter gene response is a valuable quantitative tool for the assessment of toxic potential in environmental mixtures contaminated by metals. This point is particularly relevant when considering the reduced cost of the cellular test by comparison to a battery of *in vivo* ecotoxicity tests. However, our study should be considered as a first step in the application of such *hsp70* reporter gene test and more data will be necessary to confirm our findings and validate its use in environmental biomonitoring.

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Table 1 : Wastes description according to the European Waste Catalogue.

Waste number	Waste description (industrial sector)
1	Sludge from on-site effluent treatment containing chromium (leather and fur industry)
2	Sludge from on-site effluent treatment containing chromium (leather and fur industry)
3	Waste from treatment of salt slags and black drosses (aluminium thermal metallurgy)
4	Rolling-mill sludge (iron and steel industry)
5	Sludge from gas treatment (iron and steel industry)
6	Waste from the processing of slag (iron and steel industry)
7	Solid waste from gas treatment of electrical furnaces (iron and steel industry)
8	Furnace dust (casting and ferrous pieces)
9	Ball mill dust (aluminium metallurgy)
10	De-inking sludge (paper recycling)
11	Flue gas dust (second smelting, waste from aluminium metallurgy)
12	Waste from paint powders (manufacture, formulation, supply and use of paints and varnishes)
13	Tank bottom sludge (petroleum refining)
14	Waste from dried paints (manufacture, formulation, supply and use of paints and varnishes)

Table 2 : Standardised ecotoxicity tests.

Organisms	Species	Abbreviation	Endpoint	Exposure time	Standard procedure
Aquatic					
Micro algae	*Pseudokirchneriella subcapitata*	ALG	Growth	72 hrs	NF EN 28692
Invertebrate	*Ceriodaphnia dubia*	CER	Reproduction	7 days	NF T 90-376 [a]
Invertebrate	*Daphnia magna*	DAP	Immobilisation	48 hrs	NF EN ISO 6341
Bacteria	*Vibrio fischeri*	VIB	Luminescence inhibition	30 min	NF EN ISO 11348
Terrestrial					
Earthworm	*Eisenia fetida*	EAR	Mortality	14 days	ISO 11268-1
Lettuce	*Lactuca sativa*	LET	Emergence and growth	14 days [b]	ISO 11269-2
Barley	*Hordeum vulgare*	BAR	Emergence and growth	14 days [b]	ISO 11269-2

a test performed on Evian® water without EDTA

b 14 days after emergence of 50% of the seedlings in the control.

Table 3: Effect of wastes eluates on *hsp70* promoter induction and total protein content in HeLa-CAT cells[a].

Wastes (% vol/vol)	0	0.1	0.25	0.5	1	2.5	5	10	25	50	90
Hsp70 induction (pg CAT/mg prot.)											
1	2	4	-	-	**7**	**12**	**29**	**81**	**156**	**32**	L
2	7	-	-	-	-	-	6	**13**	**17**	**38**	**71**
3	2	-	-	-	-	-	5	9	**10**	8	**40**
4	3	-	-	-	0	-	4	1	5	4	6
5	1	-	-	-	1	-	2	3	2	**28**	**163**
6	12	-	-	-	16	13	13	12	10	10	L
7	2	-	-	6	**14**	**274**	**825**	**523**	L	L	L
8	4	6	**22**	**1711**	L	L	L	L	L	L	L
9	6	-	-	-	6	10	6	11	L	L	L
10	2	-	-	-	2	3	2	5	7	**24**	L
11	0	3	**8**	**11**	**22**	**133**	**2408**	L	L	L	L
12	8	-	-	-	-	-	-	9	8	8	**16**
13	4	-	-	-	-	-	10	10	**18**	**20**	L
14	15	-	-	-	11	15	17	19	30	**343**	L
Total protein content (% of control)											
1		94	-	-	100	111	111	105	**89**	**14**	**0**
2		-	-	-	96	109	107	105	103	**82**	**60**
3		-	-	-	100	107	107	106	111	106	**59**
4		-	-	-	102	107	110	120	120	115	**84**
5		-	-	-	96	-	97	97	106	**77**	**40**
6		-	-	-	90	115	98	89	102	103	**19**
7		100	-	97	96	**84**	**73**	**17**	**0**	**0**	**0**
8		100	103	**77**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
9		-	-	-	93	102	94	**72**	**0**	**0**	**0**
10		-	-	-	96	100	101	103	93	**72**	**0**
11		97	98	98	95	**86**	**57**	**0**	**0**	**0**	**0**
12		-	-	-	106	102	111	105	117	116	**86**
13		-	-	-	99	96	102	102	102	112	**0**
14		-	-	106	108	100	100	91	**68**	**28**	**0**

a : significant effect of eluate exposure versus control cultures are in boldface ($p<0.05$, mean values of 3 wells per concentration) ; - : not tested ; L: total cellular lysis.

Table 4 : Metal contents (in mg.l^{-1}) of the wastes eluates.

Metals	Wastes[a]										
	1	2	3	4	5	6	7	8	9	11	14
As	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Cd	<0.005	0.005	<0.005	<0.005	<0.005	<0.005	2.72	8.86	<0.005	0.65	<0.005
Cr	0.03	0.09	<0.01	<0.01	<0.01	<0.01	0.02	-	<0.01	<0.01	<0.01
Cr(VI)	<0.01	<0.01	-	-	-	<0.01	<0.01	<0.01	<0.01	-	<0.01
Cu	-	-	<0.005	0.005	<0.005	<0.005	0.01	0.035	0.06	1.3	0.015
Hg	<0.001	<0.001	<0.001	-	-	-	-	<0.001	-	0.006	-
Ni	0.02	0.14	<0.02	<0.02	0.035	<0.02	<0.02	0.14	0.04	0.07	<0.02
Pb	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	0.05	9.96	<0.02	0.5	0.04
Sn	-	-	<0.05	<0.05	<0.05	<0.05	<0.05	0.38	<0.05	<0.05	0.09
Zn	0.06	<0.01	<0.01	<0.01	2.85	<0.01	1.43	1135	<0.01	0.2	0.08

a : Metal contents of wastes 10, 12 and 13 are not available. - : not analysed.

Table 5 : Ecotoxicity and *hsp70* induction potencies of the 14 wastes.

Waste	*In vivo* toxicity (EC_{20} in % sample) [a]							*hsp70* induction	
No.	BAR	LET	EAR	ALG	DAP	VIB	CER	PI[b]	X_{slope}
1	4.3	2.0	4.2	1.1	9.2	0.6	1.0	6.83	8.5
2	6.9	0.7	14.5	2.6	13.3	0.2	7.7	0.69	41.0
3	58.0	4.4[c]	17.5	23.9	NT	NT	50.5	0.44	74.3
4	18.6	16.1	NT	NT	NT	NT	84.1	0.1[d]	NI
5	NT	NT	NT	2.2	69.1	36.6	0.3	2.02	85.9
6	85	45.3	85.3	NT	NT	NT	48.9	0.1[d]	NI
7	1.8	1.3	1.6	0.6	5.1	17	0.006	158.81	3.9
8	3.5	1.0	1.2	0.0004	0.1	0.02	0.004	3320.12	0.4
9	16.9	6.3	3.3	3.3	5.3	8.7	0.2	0.1[d]	NI
10	NT	75	NT	63.6	37.0	5.4	11.7	0.40	28.2
11	1.0	0.2	0.5	0.7	11.0	3.9	0.1	469.60	3.8
12	47.5	22.6	NT	65.7	88.7	NT	>70	0.20	72.6
13	1.5	0.68[c]	1.0	35.5	5.4	3.3	0.6	0.24	7.4
14	2.0	2.1	8.6	0.8	11.8	2.4	0.1	7.87	37.8

a : NT, not toxic ; the test codes are given in the Table 2;

b : *hsp70* induction potency (PI) in pgCAT/mg protein/sample concentration unit;

c : effect measured on emergence;

d : for non effective samples, a PI of 0.1 (*i.e.* 10 pg CAT/mg at the 100% concentration), corresponding to the control background, is given.

Table 6 : Pearson's correlation coefficients between *hsp70* induction parameters and *in vivo* bioassays (EC_{20}) [a].

		CER	ALG	DAP	VIB	BAR	LET	EAR
PI	r	-0.816	-0.868	-0.711	-0.597	-0.623	-0.574	-0.655
	p	0.000	0.000	0.004	0.024	0.011	0.032	0.011
	n	*14*	*14*	*14*	*14*	*14*	*14*	*14*
EC_{slope}	r	0.731	0.777	0.847	0.651	0.693	0.651	0.763
	p	0.003	0.001	0.000	0.012	0.006	0.012	0.002
	n	*14*	*14*	*14*	*14*	*14*	*14*	*14*

a All data were included into the correlation. The test codes are given in the Table 2. r = correlation coefficients ; p = signification level ; n = number of samples fitted into the correlation.

Table 7 : Pearson's correlation coefficients between *hsp70* induction parameters and *in vivo* bioassays (EC_{20}) [a].

		CER	ALG	DAP	VIB	BAR	LET	EAR
PI	r	-0.864	-0.882	-0.730	-0.344	-0.563	-0.538	-0.612
	p	0.001	0.000	0.016	0.364	0.115	0.109	0.107
	n	*10*	*11*	*10*	*9*	*9*	*10*	*8*
EC_{slope}	r	0.759	0.765	0.910	0.545	0.628	0.517	0.818
	p	0.011	0.006	0.000	0.129	0.070	0.126	0.013
	n	*10*	*11*	*10*	*9*	*9*	*10*	*8*

a Correlation were performed on the toxic data only. The test codes are given in the Table 2. r = correlation coefficients ; p = signification level ; n = number of samples fitted into the correlation.

Figure 1 : Examples of modelled dose-response curves of *hsp70* promoter induction and cytotoxicity by wastes eluates.

Plots : measured *hsp70* induction (in ng CAT/mg protein, refers to the left ordinate axis) ; heavy lines : calculated induction ; light lines : calculated cytotoxicity (in % of control, refers to the right ordinate axis).

Figure 2 : Linear regression between *hsp70* induction potency (PI) and metals contents (µg/l) of the samples. Dashed lines represent 95% confidence intervals.

Figure 3 : Position of wastes (A) and toxicity tests (B) on the first factorial plane of the principal component analysis.

Figure 4 : Correlation between aquatic tests and *hsp70* induction. PI was plotted *vs* (A) *C. dubia*, (B) *R. subcapitata* and (C) *D. magna* tests results. Dashed lines represent 95% confidence intervals.

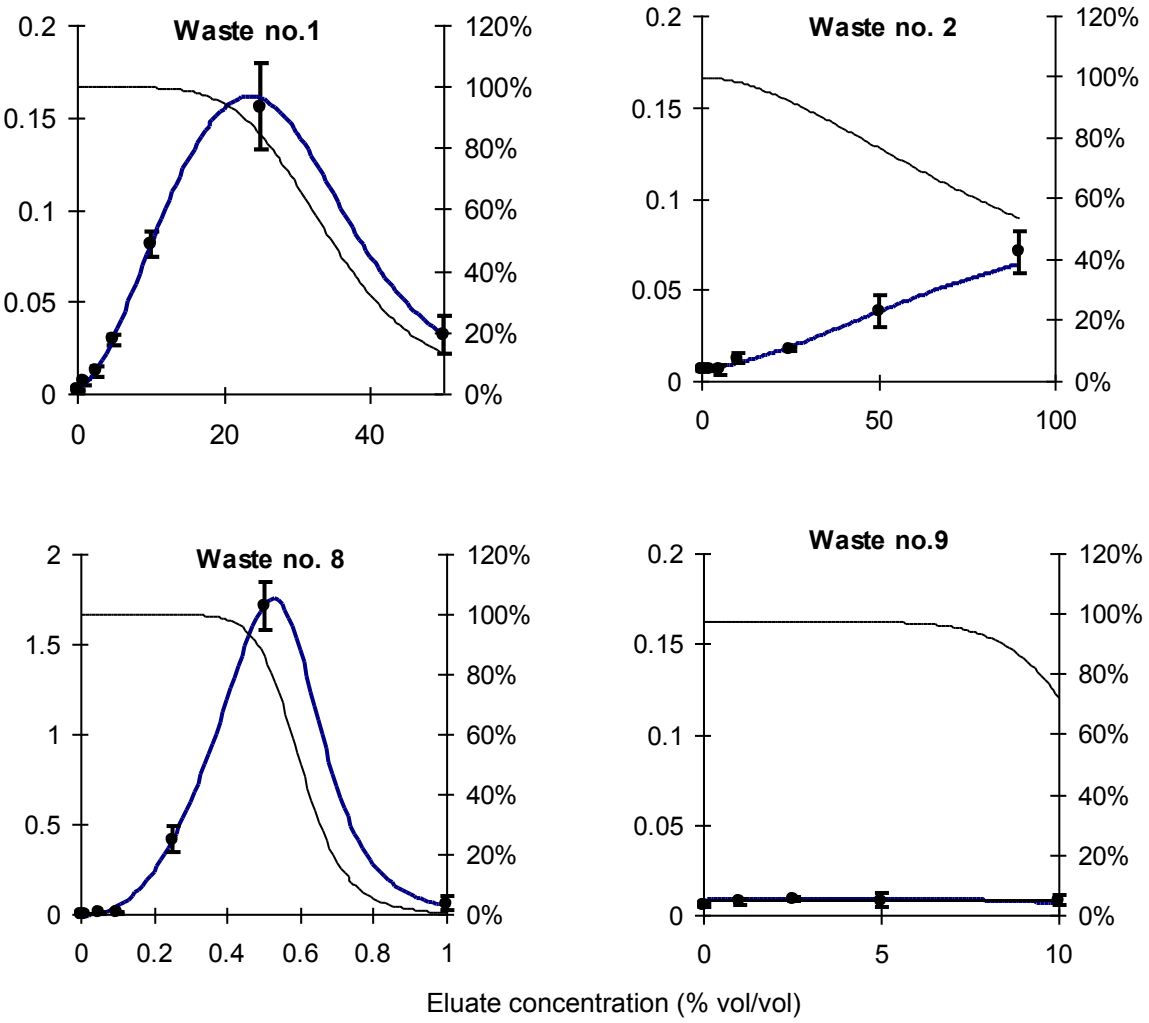

Waste no.1
Waste no. 2
Waste no. 8
Waste no.9
Eluate concentration (% vol/vol)

Figure 1

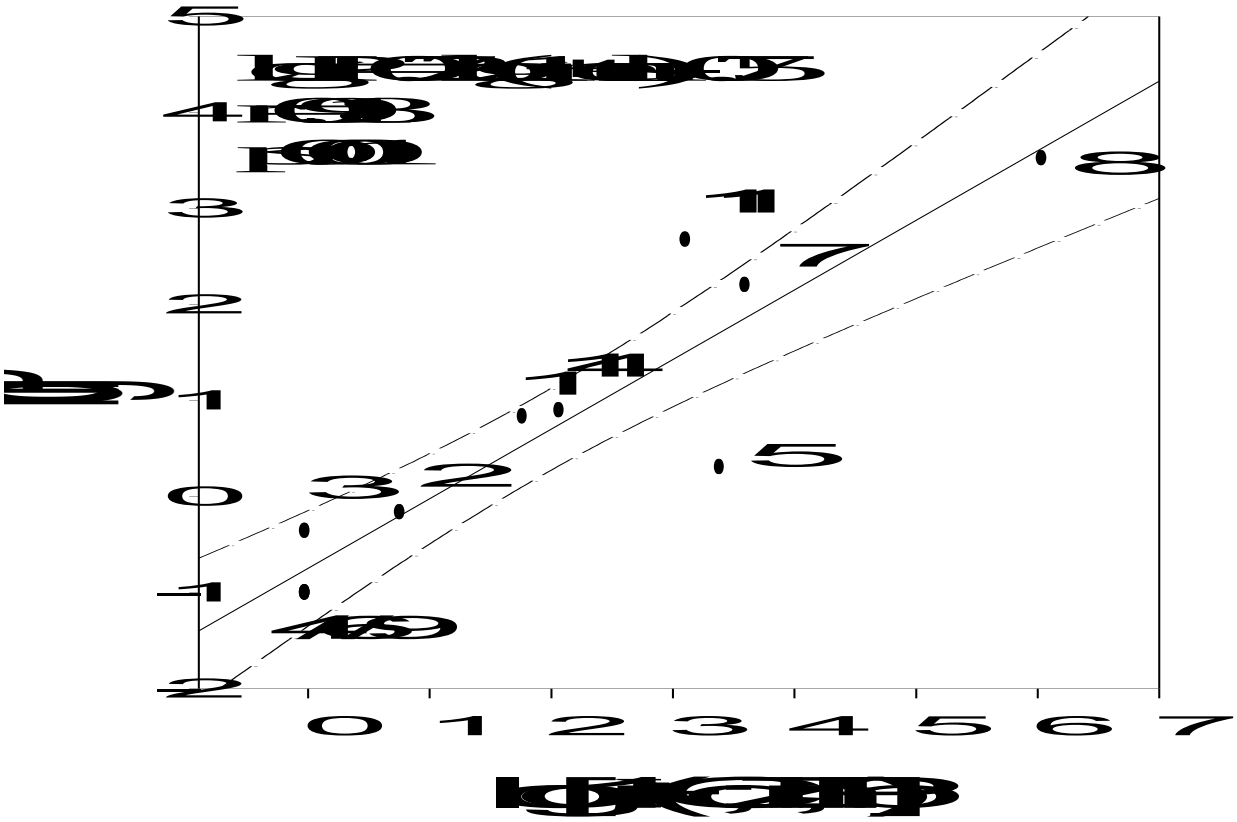
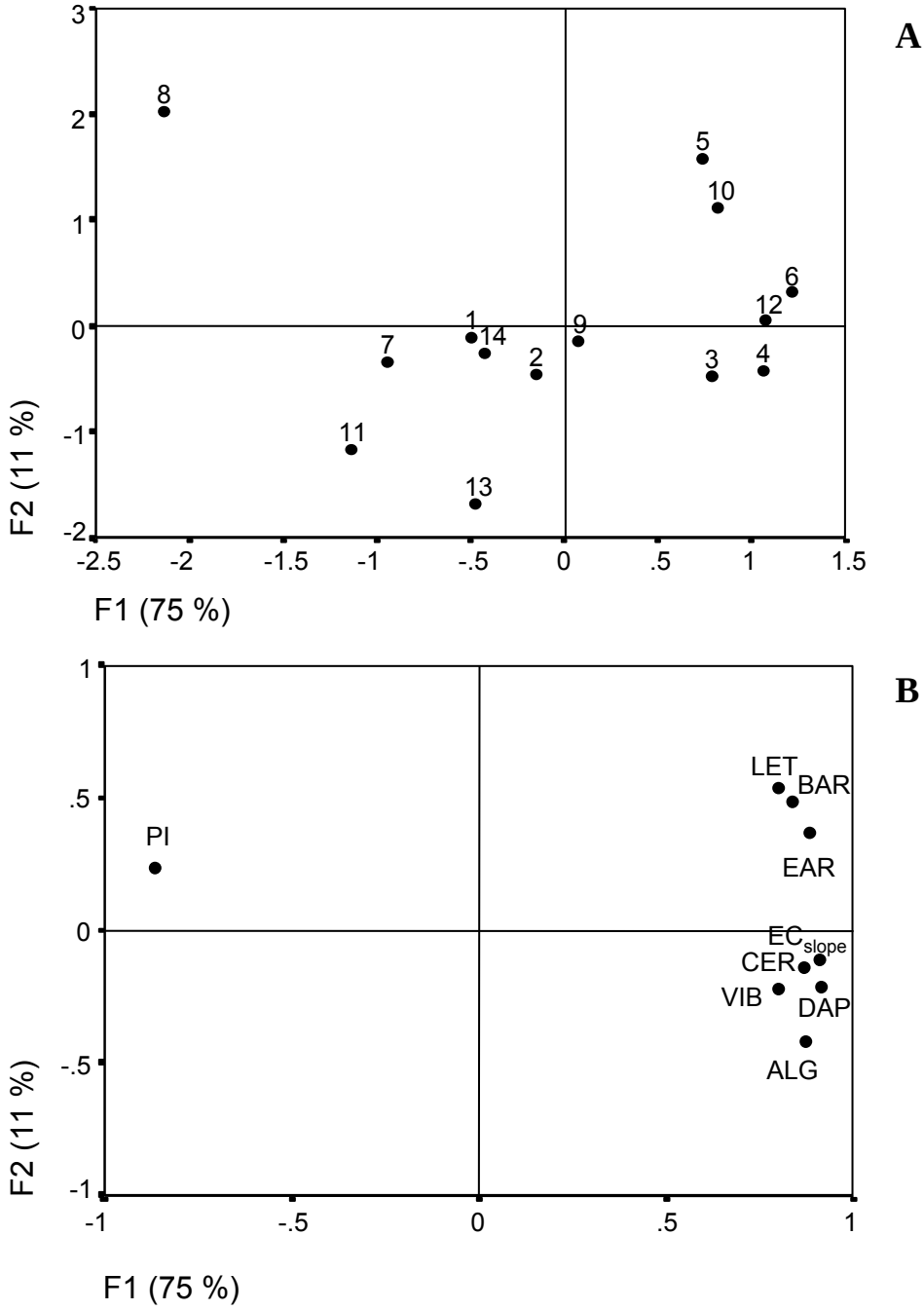


Figure 2


A
3
2
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0
-1
-2
F2 (11 %)
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F1 (75 %)
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-1
F2 (11 %)
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Figure 3

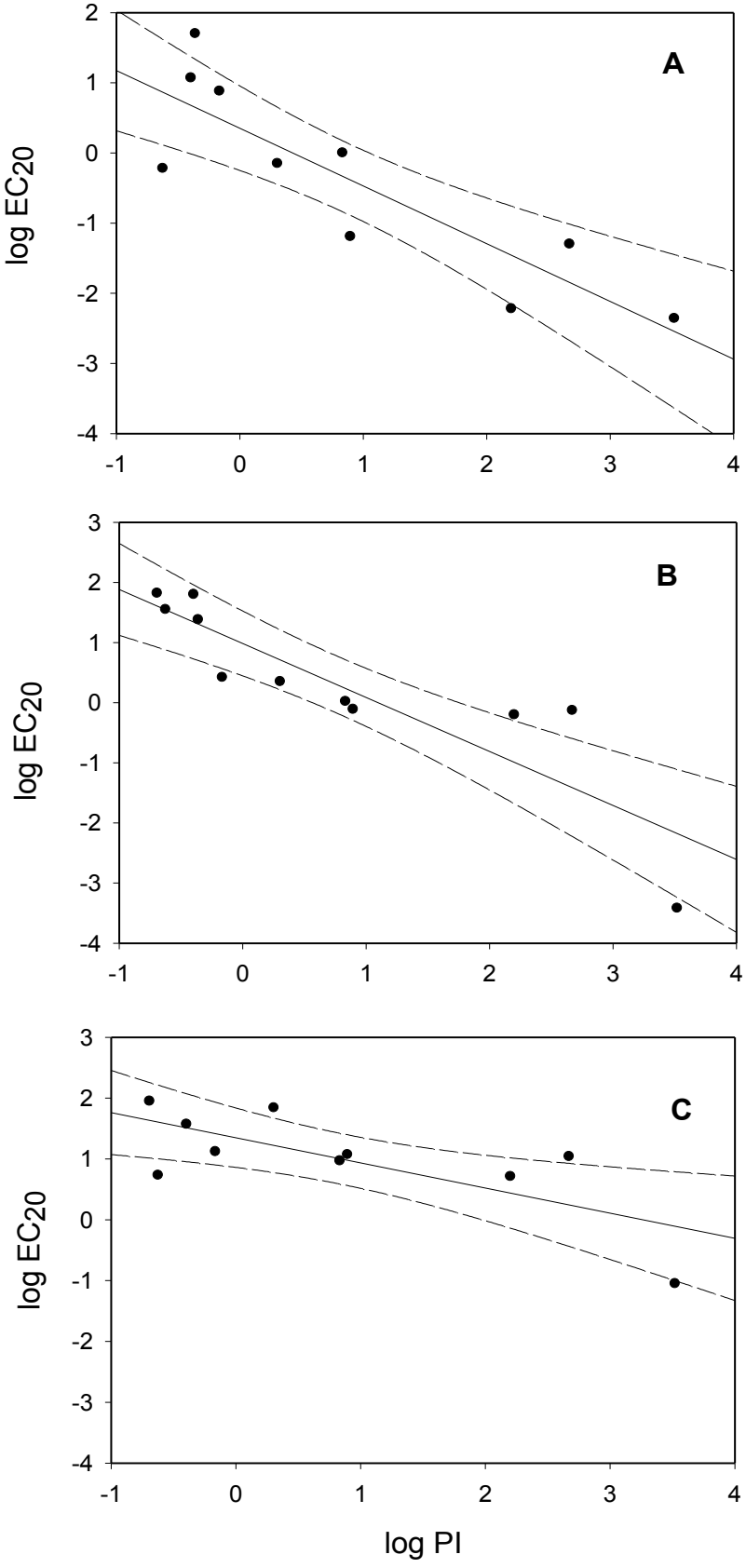

Figure 4